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Short communication

Experimental nebulization of American bison (*Bison bison*) with low doses of ovine herpesvirus 2 from sheep nasal secretions

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ABSTRACT

Malignant catarrhal fever (MCF), caused by ovine herpesvirus 2 (OvHV-2), is an important cause of mortality in ranched American bison and domestic cattle in North America. Previous studies showed that bison can be infected by intranasal nebulization with sheep nasal secretions containing OvHV-2 and provided preliminary information on viral doses required for infection and disease progression. The goals of this study were to establish optimal minimal infectious and minimal lethal doses of OvHV-2 by the intranasal route in bison, evaluate the influence of dose on incubation period and other clinical parameters and determine if bison seropositive for antibody against MCF-group viruses are resistant to developing MCF after intranasal challenge. In this study, the minimal infectious dose and minimal lethal dose overlap, suggesting that experimental production of subclinically infected bison is impractical. Dose is inversely related to both incubation period and the period between nebulization and first detection of >1000 OvHV-2 DNA copies/500 ng total DNA in peripheral blood leukocytes. Interestingly, all of the bison seropositive for anti-MCF-group viral antibody prior to inoculation died of MCF after nebulization. We conclude that previous exposure to an MCF-group virus does not necessarily provide resistance to OvHV-2-induced MCF in bison.

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1. Introduction

Malignant catarrhal fever (MCF) is an acute, generally fatal disease of ungulates that is an important cause of mortality in American bison (*Bison bison*) as well as domestic cattle in the United States and Canada (Li et al., 2006; Moore et al., 2009). Outbreaks of MCF in bison have resulted in severe losses for bison producers (Li et al., 2006; Schultheiss et al., 2000). The disease in bison and cattle in North America is almost always caused by ovine herpesvirus 2 (OvHV-2), which is a member of a group of ruminant gammaherpesviruses in the *Macavirus* genus referred to as the MCF-virus group (Davison et al., 2009; Li et al., 2005). These viruses are carried persistently and subclinically in well-adapted host species and can cause widespread vasculitis, lymphoproliferation and epithelial necrosis when poorly adapted host species become infected (Russell et al., 2009). Sheep are the reservoir for OvHV-2. Recently it was shown that although OvHV-2-induced MCF in bison is usually fatal once clinical signs develop, subclinical infection of bison does occur. In one feedlot, 23.7% of the bison were seropositive for antibody against MCF-group viruses and 11.3% of those had detectable OvHV-2 DNA in the peripheral blood (O'Toole et al., 2002). Herpesvirus infections are lifelong and

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therefore these animals are considered persistently infected.

Because OvHV-2 has never been propagated in culture, a method of intranasal nebulization with nasal secretions collected from sheep during shedding episodes (Li et al., 2004) is used for experimental inoculation of sheep, bison, and cattle (O'Toole et al., 2007; Taus et al., 2005, 2006). Initial nebulization studies in bison defined the OvHV-2 doses required for infection and for inducing disease (O'Toole et al., 2007). Because of the small numbers of animals in each dose group and relatively large differences between dose groups, further dose response experiments were needed to more precisely define the optimal subclinical dose and the minimum LD_{100} so that as few animals as possible would be required for future vaccine and pathogenesis studies. The dose response portion of this study was also intended to test the hypothesis that dose affects incubation period and other measurable clinical parameters associated with viral infection dynamics and disease progression.

Although little is known about the immune response in OvHV-2 infected bison, induction of protective immunity has been documented within the MCF-virus group, specifically with alcelaphine herpesvirus 1 (AlHV-1), another MCF-group virus persistently carried by wildebeest. Plowright (1968) reported experiments with cattle that were subclinically infected or had survived AlHV-1-induced MCF. Challenge with virulent AlHV-1 was repeated eight times over a 4-year period and none of the cattle subsequently developed MCF. More recently, vaccination with an attenuated strain of AlHV-1 induced partial protection against intranasal challenge with virulent AlHV-1 (Haig et al., 2008). For this study, we hypothesized that subclinical infection of bison with an MCF-group virus would result in protection against MCF when animals were subsequently challenged intranasally with a minimal lethal dose of OvHV-2.

2. Materials and methods

A group of 16 yearling bison were purchased from a rancher and maintained at the Wyoming State Veterinary Laboratory in accordance with an approved animal care and use protocol. The bison were screened six times over a 170-day period prior to the beginning of the experiment for the presence of antibody against MCF-group viruses in plasma by cELISA as described previously (Li et al., 2001) and for the presence of OvHV-2 DNA in peripheral blood leukocytes (PBL) by semi-nested PCR using OvHV-2 specific primers (Baxter et al., 1993; Li et al., 2004). Antibody against MCF-group viruses was detected at two or more time points in six bison during the pre-inoculation period (Table 1, nos. 1–4, 8 and 16) and OvHV-2 DNA was detected in two bison at one time point each (nos. 1 and 2). All samples that were seropositive prior to nebulization were tested for other herpesviruses using consensus primers that amplify a segment of the herpesviral DNA polymerase gene (VanDevanter et al., 1996).

The inoculum used for intranasal nebulization was collected and prepared as previously described (Li et al., 2004). The OvHV-2 DNA copy number was determined by real-time PCR (Hussy et al., 2001; Li et al., 2004) and the inoculum used for this study is the same used to inoculate sheep, cattle and bison in previously reported experiments

Table 1
Clinical and laboratory findings in bison inoculated with OvHV-2 by intranasal nebulization.

Group	Inoculation dose[a]	Animal no.	Antibody[b]	DNA[b]	Clinical signs[b]	Death[b]	Cause of death[e]
A	1×10^5	1	−127[c]	−127[c] (49)[d]	54	54	MCF
		2	−127	−127 (35)	48	49	MCF
		3	−127	28 (35)	48	49	MCF
B	4.5×10^4	4	−72	21 (35)	48	49	MCF
		5	35	35 (42)	56	56	MCF
		6	–	49 (77)	87	87	MCF
C	1.4×10^4	7	49	28 (35)	50	54	MCF
		8	−16	28 (35)	35	36	Emaciation[f]
		9	–	35 (35)	None	36	Accident[f]
		10	–	–	None	124	Trauma
D	5×10^3	11	35	35 (56)	65	65	MCF
		12	–	–	36	38	Unknown
		13	–	56	na	na	na
		14	–	–	na	na	na
E	Neg. control	15	7	–	na	na	na
		16	−16	–	na	na	na

[a] Inoculation dose reported as OvHV-2 DNA copies in sheep nasal secretions detected by real-time PCR. The negative control group was nebulized with nasal secretions from OvHV-2 negative sheep.

[b] Reported as days post-inoculation (PI) of first detection (antibody and DNA) or the day the animal was found dead, died naturally or was euthanized. (–) = not detected, na = still alive at end of study.

[c] Negative numbers indicate detection of antibody or DNA prior to inoculation.

[d] The numbers in parentheses indicate the day PI that >1000 copies OvHV-2 DNA/500 ng total DNA were first detected in PBL by real-time PCR. Where there are no parentheses, copy number remained below 1000.

[e] Cause of death was determined by gross and histologic examination of tissues along with PCR results. Unknown = no gross or histologic lesions consistent with MCF and cause of death was not identified, na = still alive and healthy at end of study.

[f] Animals had no gross or histologic lesions suggestive of MCF but had 3480 (no. 8) and 2300 (no. 9) OvHV-2 DNA copies in the PBL the day before death and had OvHV-2 DNA in multiple tissues indicating they probably would have gone on to develop MCF.

(O'Toole et al., 2007; Taus et al., 2005, 2006). The bison were nebulized as previously described in four dose groups (Table 1) between the non-infectious dose (1×10^3 OvHV-2 DNA copies) and LD_{100} (1×10^5 OvHV-2 DNA copies) from the earlier study (O'Toole et al., 2007). Two bison were nebulized with nasal secretions from OvHV-2 negative sheep (Group E). Bison seropositive prior to inoculation were randomly assigned to the higher dose groups so that survival could potentially be attributed to resistance rather than having received a non-lethal dose of virus.

After nebulization, blood was collected weekly for 12 weeks, monthly for 4 months and then intermittently until the end of the study at 9 months PI. All samples were tested for MCF-group viral antibody and for OvHV-2 DNA by semi-nested PCR. Real-time PCR was performed on all PBL samples that were positive by semi-nested PCR and on selected tissue samples. All real-time PCR results from PBL and tissues are reported as OvHV-2 DNA copies per 500 ng total DNA. Necropsies were done on all animals that were either found dead or were euthanized due to clinical signs, and samples of selected tissues were collected and stored at −80 °C or were fixed in 10% neutral buffered formalin and processed routinely for histopathology.

An unpaired *t*-test was used to analyze the relationship between viral dose and incubation period, between viral dose and the time from challenge to detection of >1000 OvHV-2 copies in PBL and between viral dose and the interval from first detection of >1000 OvHV-2 copies in PBL to onset of clinical signs. Data from bison with confirmed MCF in the current study (Table 1; nos. 1–7 and 11) were combined with data from two other studies in our laboratory that utilized the same inoculum pool and the same nebulization method. One study has been published (O'Toole et al., 2007) and data included here is from MCF-affected bison that had been nebulized with the following doses: 1×10^4 ($n = 1$), 1×10^5 ($n = 2$), 1×10^6 ($n = 2$), 1×10^7 ($n = 2$), 2.7×10^7 ($n = 2$), and 4.7×10^7 ($n = 2$). Data included from the third study (unpublished data) is from six MCF-affected bison nebulized with 1×10^7 OvHV-2 DNA copies. Two bison from the current study died of conditions other than MCF but had >1000 copies in the PBL (nos. 8 and 9, see Section 3) and were included in the analysis of the period from challenge to rise in viral DNA in the PBL but not in the other two analyses. The data were divided into two groups: bison nebulized with $\leq 1 \times 10^5$ OvHV-2 DNA copies ($n = 11$ or 13) and bison nebulized with $\geq 1 \times 10^6$ OvHV-2 DNA copies ($n = 14$). Approximately equal percentages of animals in both dose groups where seropositive prior to nebulization (42.9% in the high dose group and 38.5% in the low dose group) making it unlikely that serostatus affected the results.

3. Results and discussion

The serological data, PCR results and clinical outcome are indicated in Table 1. All eight of the bison that died of MCF (Table 1, nos. 1–7 and 11) had between 7500 and 326000 OvHV-2 copies in the PBL within 24 h of death (data not shown). One bison (no. 8) died on day 36 PI after a short history of weakness and incoordination and was diagnosed with emaciation of unknown cause at necropsy. Another bison (no. 9) also died on day 36 PI after accidental entanglement in the feed bunk. Both of these bison remained PCR negative until the day before they died, when 3480 and 2300 OvHV-2 copies were detected in PBL, respectively. Neither had gross or histologic lesions suggestive of MCF but both had up to 2570 OvHV-2 DNA copies in tissues. OvHV-2 DNA levels in the PBL and tissues of all subclinically infected bison tested in our laboratory to date (that did not go on to develop MCF) have been below the detection limit of the real-time PCR assay (O'Toole et al., 2007, unpublished data). Thus, we consider it likely that these two bison were in the preclinical stage and would have gone on to develop MCF had they not died of unrelated causes. Bison no. 12 died on day 38 PI after a short period of lethargy and bison no. 10 died 18 weeks after inoculation due to accidental traumatic vertebral dislocation. Both of these animals were seronegative, had no gross or histologic lesions consistent with MCF and had no detectable OvHV-2 DNA in the PBL or tissues. OvHV-2 DNA was detected in a single PBL sample from one of the two other inoculated bison (no. 13) on day 56 PI and this animal remained healthy and was considered subclinically infected. The two negative control animals (nos. 15 and 16) and the other inoculated bison (no. 14) remained PCR negative and healthy for the duration of the study period.

Although there have been many OvHV-2-induced MCF cases in cattle, it is clear that bison are much more clinically susceptible (O'Toole et al., 2007; Taus et al., 2006). The ability to consistently produce bison subclinically infected with OvHV-2 would significantly advance research efforts related to host immunity and vaccine development and therefore, one goal of this study was to define the intranasal dose of OvHV-2 in bison that would most efficiently result in the establishment of persistent subclinical infection. Nebulization with 5×10^3 OvHV-2 DNA copies resulted in one persistent subclinical infection and one case of MCF, indicating that the minimum infectious dose and the minimum lethal dose were in a very narrow range in this study. Consequently, experimental production of subclinically infected bison is unlikely to be efficient and animals infected under natural field conditions are currently the only practical source of subclinically infected bison. All bison inoculated with 4.5×10^4 OvHV-2 DNA copies died of MCF and this was the minimum LD_{100} in this study.

The combined data from this and two previous studies confirm a statistically significant relationship between dose and incubation period as well as a relationship between dose and time to a rise in viral DNA copy number in PBL in bison (Fig. 1A and B). However, there was no significant difference between the two dose groups in terms of time from rise in OvHV-2 DNA in PBL to development of clinical signs (Fig. 1C). These data suggest that dose plays a critical role in determining the initial timeline of OvHV-2 infection in bison but that once a threshold level of viral DNA is reached in the peripheral blood, the infection progresses to disease at a relatively constant rate. Thus, measuring viral DNA levels in PBL can be used to predict onset of clinical signs. No relationship was detected between dose and peak OvHV-2 DNA copy

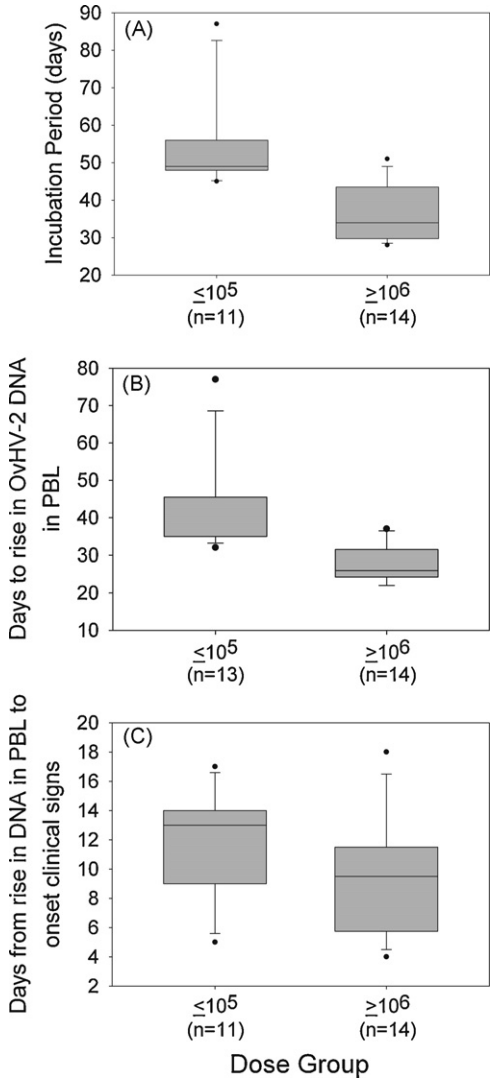

Fig. 1. Statistical analysis of the relationship between dose and clinical parameters. Data are from animals in the current study combined with data from a previously published study (O'Toole et al., 2007) and another study in our laboratory (see Section 2). Data are divided into two dose groups based on whether the inoculation dose was $\leq 1 \times 10^5$ or $\geq 1 \times 10^6$ OvHV-2 DNA copies. The difference in means between the two dose groups is significant for (A) incubation period (difference between means: 17.5 days; $P < 0.001$, 95% confidence interval 9.2–25.7) and for (B) days to first detection of >1000 OvHV-2 copies in PBL (difference between means: 13.8 days; $P < 0.001$, 95% confidence interval 6.2–21.4), but is not significant for (C) time from first detection of >1000 OvHV-2 copies to onset of clinical signs ($P = 0.234$; 95% confidence interval −1.3 to 5.1). The mean time from rise in OvHV-2 DNA in PBL to onset of clinical signs for all bison was 10.4 days (SD ±3.9).

number in PBL or between dose and gross or histologic lesions.

Another objective of this study was to determine whether bison that are seropositive for antibody against MCF-group viruses (and thus persistently infected with an MCF-group virus) are resistant to developing MCF after intranasal challenge with a dose of OvHV-2 that is consistently lethal in seronegative bison. Our original plan was to use bison that became subclinically infected during the dose response experiment as subjects for a subsequent challenge study, but only one bison became subclinically infected; therefore, the additional study was not pursued. However, conclusions are possible from analysis of data from five bison that were seropositive prior to nebulization, two of which had OvHV-2 DNA in the PBL (Table 1, nos. 1–4, 8, 16). Four of these bison (including the two PCR positive animals) died of confirmed MCF (nos. 1–4) and the other (no. 8) died of unrelated causes but with significant levels of OvHV-2 DNA in PBL and tissues, suggesting that it probably would have gone on to develop MCF. Thus, the negative control animal was the only bison that was seropositive prior to nebulization that did not have confirmed MCF or any indications that it might develop MCF.

Failure to detect OvHV-2 DNA in peripheral blood of four of the seropositive bison prior to nebulization may be due to low levels of viral DNA or to possible strain differences (as yet unstudied) that could affect PCR specificity. Another possibility that cannot be ruled out is that some bison are subclinically infected with another MCF-group virus resulting in seroconversion but negative OvHV-2 specific PCR. However, this is speculation and to our knowledge no such viruses have been reported and none were detected in this study using herpesvirus consensus PCR (data not shown). Nevertheless, it is meaningful that at least two bison known to be infected with OvHV-2 based on semi-nested PCR showed no resistance and that the remainder of the bison with a detectable antibody response against this group of viruses (and thus persistently infected with an MCF-group virus) showed no indication that the persistent subclinical infection resulted in protective adaptive immunity to a virus within the same group.

Cross-protective immunity among closely related herpesviruses does occur, most notably, between viruses within the *Gammaherpesvirinae* subfamily. Cattle immunized with bovine herpesvirus 4 (BoHV-4), a non-MCF-group gammaherpesvirus (Davison et al., 2009) that is serologically cross-reactive with AlHV-1, were partially protected against MCF when challenged with a virulent strain of AlHV-1 (Rossiter et al., 1988). Cross-protective immunity within the *Alphaherpesvirinae* subfamily is also well documented. Vaccination with nonpathogenic turkey herpesvirus (maleagrid herpesvirus 1; genus *Mardivirus*) has been used for many years to protect chickens against Marek's disease, a lymphoid neoplasm caused by two viruses in the same genus (gallid herpesvirus 2 and 3) (Davison et al., 2009; Gimeno, 2008). A reasonable inference from this study is that some of the bison may have been infected with an MCF-group virus other than OvHV-2 but that, unlike in the cases of AlHV-1 and BoHV-4 or Marek's disease, there is not a sufficient cross-reactive immune response between viruses within the MCF-virus group to protect against developing the disease. Alternatively, it is possible that seropositive bison were subclinically infected with OvHV-2 and their immune response was insufficient or of the incorrect type to prevent disease upon challenge. If subclinical infection with an MCF-group virus can provide protection against OvHV-2-induced MCF in bison it may only occur in

individual animals that have certain advantages such as differences in genetically coded aspects of the immune system, other inherent factors affecting susceptibility to infection or health status at the time of initial infection or challenge. An association between MHC class II haplotype and resistance to MCF has been reported (Traul et al., 2007), although the influence of MHC class II haplotype in this study is unknown. Future studies will be designed to elucidate the details of disease progression and pathogenesis as well as to define the host immune response and factors that affect it in order to ultimately develop ways to control this important disease.

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